Characterization of porcine-specific surface (S-) layer protein carrying *Lactobacillus* species, S-layer proteins and the adhesin of *Escherichia coli* F18 fimbriae

– potential applications for veterinary medicine

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ABSTRACT

Pigs coexist with diverse and dense commensal microbiota in their gastrointestinal tract (GIT). Lactobacilli, identified as common members of porcine intestinal microbiota, have been considered to be an important group of bacteria in maintaining the stability of GIT, in preventing intestinal infections and generally, in supporting intestinal health. Because several species of lactobacilli have GRAS (generally regarded as safe) status and some of them have an ability to interact with intestinal epithelial cells, thus promoting host-bacterial interactions, their possible applications as mucosal vaccine vector and/or probiotics have aroused interest.

Selection criteria for lactobacilli to be used as vaccine vector or probiotic include the abilities to adhere to the intestinal epithelium cells and colonize the lumen of the GI tract. The adherence to host tissues, which enables the organism to overcome local defences such as mucociliary function and peristaltis, is usually mediated by specialized proteins called adhesins. These adhesins are responsible for recognizing and binding to specific receptor structures of the host cells. Bacterial adhesins are often found in hair-like appendages called pili or fimbriae that extend outward from bacterial surface. Alternatively, they can be directly associated with the microbial cell surface.

Surface layer proteins (Slps) of lactobacilli have been shown to confer tissue adherence. In this study, S-layer positive lactobacilli from the intestine and faeces of pigs were isolated and their ability to adhere to pig and human intestinal cells as well as to extracellular matrix (ECM) components, collagen, laminin and fibronectin were studied. The adherence of S-layer carrying lactobacilli varied from strong to moderate for human and porcine small intestine enterocytes and for the components of ECM and basal membranes (BM). Removal of the intact Slps reduced the adhesion of some strains to fibronectin and laminin, whereas, the adhesiveness to laminin increased with some strains.

Besides the putative binding properties of Slps, a very large number of surface layer protein subunits present in an S-layer make the use of the S-layer structure a very

interesting alternative to surface display antigens. Therefore, the aim of this study was to characterize the S-layer proteins. Two new surface layer proteins (SlpB and SlpD), with potential to be tested as antigen carriers, were characterized, and three S-layer protein (*slpB, slpC* and *slpD)* genes were isolated, sequenced, and studied for their expression in *Lactobacillus brevis* neotype strain ATCC 14869. Under different growth conditions, *L. brevis* strain was found to form two colony types, smooth (S) and rough (R), and to express the S-proteins differently by mechanism not involving DNA rearrangements. The adhesion studies indicate that *L. brevis* adheres to human and pig intestinal epithelial cells but it is not currently known whether the binding is mediated by the surface proteins in this *L. brevis* strain of human origin.

To identify the S-layer positive lactobacilli strains used in this study, a polyphasic taxonomic approach was applied. The methods used included 16S rRNA gene sequence analysis, numerical analysis of 16 and 23 rRNA gene ribotypes and DNA-DNA reassociation. In addition, all strains were included in a multilocus sequence analysis (MLSA) study for species identification using housekeeping genes encoding the phenylalanyl-tRNA synthase alpha subunit (*pheS*) and RNA polymerase alpha subunit (*rpoA*). These results indicated that six out of eight porcine lactobacilli strains from Finland and the related *L. sobrius* strains, originating from porcine intestine from elsewhere, constitute a single species, *L. amylovorus*, and that the name *L. sobrius* should be considered as a later synonym of *L. amylovorus*.

In the final part of this study, the adhesin FedF of *Eschrichia coli* F18 fimbriae was characterized. The work aims at developing lactobacilli as a live mucosal vaccine vector for pigs against diseases caused by $F18^{+}$ *E. coli*. The F18 fimbriae carrying *E. coli* strains colonize the microvilli of porcine small intestinal epithelial cells and cause post-weaning diarrhoea (PWD) and edema disease (ED) in pigs. It has been shown that oral immunization of weaned piglets with adhesins can induce a protective mucosal immune response. Naked FedF appeared to be very unstable but in our study it could be produced as a fusion protein with maltose binding protein (MBP). Specific adhesion to isolated porcine intestinal epithelial cells was demonstrated with MBP-FedF fusions as

well as the ability of anti-MBP-FedF antibodies to prevent binding of *E. coli* F18 to porcine epithelial cells.

ABBREVIATIONS

aa	amino acids
ATCC	American Type Culture Collection
BM	basal membrane
CE	competitive exclusion (so called Nurmi concept)
DNA	deoxyribonucleic acid
ECM	extracellular matrix
ED	edema disease
EM	electron microscopy
ETEC	enterotoxigenic *Escherichia coli*
$F18^+$	F18 fimbriae-expressing
FITC	fluorescein isothiocyanate
FOS	fructooligosaccharides
FUT1	gene encoding the alpha (1,2)-fucosyltransferase (FUT1)
GHCl	guanidine hydrochloride
GI	gastrointestinal
GIT	gastrointestinal tract
GRAS	generally regarded as safe
kb	kilobase
kDa	kilodalton
LAB	lactic acid bacteria
MBP	maltose binding protein
MLSA	multilocus sequence analysis
MW	molecular weight

ORF, *orf*	open reading frame
PCR	polymerase chain reaction
pheS	gene encoding phenylalanyl-tRNA synthase alpha-subunit
PWD	post-weaning diarrhoea
RBS	ribosome binding site
RNA	ribonucleic acid
rpoA	gene encoding DNA-directed RNA polymerase alpha-subunit
SCWP	secondary cell wall polymers
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
S-layer	surface layer
slp	gene encoding surface layer protein
Slps	surface layer proteins
spp.	species
VTEC	verotoxigenic *Escherichia coli*

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numeral. The original articles are reprinted with the kind permission of the publishers.

I **Jakava-Viljanen, M. and A. Palva.** 2007. Isolation of surface (S) layer protein carrying *Lactobacillus* species from porcine intestine and faeces and characterization of their adhesion properties to different host tissues. Vet. Microbiol. 124: 264–273.

II **Jakava-Viljanen, M., A. Murros, A. Palva and J. Björkroth.** 2007. *Lactobacillus sobrius* Konstantinov et al. 2006 is a later synonym of *Lactobacillus amylovorus* Nakamura 1981. Int. J. Syst. Evol. Microbiol. Under revision.

III **Jakava-Viljanen, M., S. Åvall-Jääskeläinen, P. Messner, U. B. Sleytr and A. Palva.** 2002. Isolation of three new surface layer protein genes (*slp*) from *Lactobacillus brevis* ATCC 14869 and characterization of the change in their expression under aerated and anaerobic conditions. J. Bacteriol. 184: 6786–6795.

IV **Smeds, A., K. Hemmann, M. Jakava-Viljanen, S. Pelkonen, H. Imberechts and A. Palva.** 2001. Characterization of the adhesin of *Escherichia coli* F18 fimbriae. Infect. Immun. 69: 7941-7945.

1. INTRODUCTION

The intestinal commensal microbiota of pigs comprises hundreds of different types of microorganisms (Stewart, 1997; Inoue et al., 2005). The members of the genus *Lactobacillus* are important residents of the gastrointestinal (GI) microbiota and have been subjects of increasing interest due to their possible role in the maintenance of GI health. Because of these putative health promoting properties, *Lactobacillus* species are widely used as probiotics (Ouwehand et al., 2002). One important criterion proposed for a probiotic bacterium is its ability to adhere and colonize host tissues, which enables multiplication and survival of the bacterium in the host and may prevent the colonization of pathogenic bacteria via competitive exlusion (CE).

The F18 fimbriae-expressing ($F18^+$) *E. coli* cause post-weaning diarrhoea (PWD) and edema disease (ED) in newly weaned piglets. The key virulence factors in diarrhoea are enterotoxins and fimbrial adhesins (Berberov et al., 2004; Zhang et al., 2006). Protection against these diseases can be established by preventing the fimbrial adhesion of these bacteria to the enterocytes of the porcine intestine. The FedF protein of F18 fimbriae was recognised as the adhesin and antibodies against FedF were found to inhibit the adhesion of $F18^+$ *E. coli* to porcine enterocytes (Smeds et al., 2001; Smeds et al., 2003). However, oral immunisation with purified F18 fimbriae did not result in protection against ED by a challenge infection with $F18^+$ verotoxigenic *E. coli* (VTEC) (Verdonck et al., 2007) and there are no commercial vaccines available against infections caused by F18 fimbriae-carrying *E. coli* strains.

Like many other bacteria, several species of *Lactobacillus* have a surface (S-) layer as the outermost component of the cell (reviewed in Åvall-Jääskeläinen and Palva, 2005). The function of *Lactobacillus* S-layers characterized so far is involved in mediating adhesion to different host tissues. In addition to surface layer proteins (Slps) adhesive properties, the very large number of S-layer subunits present on the cell surface has prompted research aiming at the use of S-layers as a vehicle for the delivery of biologically active compounds, such as drug molecules, antibodies, enzymes and vaccine antigens (Sleytr et al., 2007).

The accurate species identification of bacteria is of fundamental importance in the development of new mucosal vaccine vectors or probiotics. Species of *Lactobacillus* form the most numerous genus in the heterogeneous group of lactic acid bacteria (LAB). The genus contains about one hundred described species, which are subdivided by 16S rRNA analysis, DNA-DNA hybridization and other phylogenetic methods, into eight major groups. The 16S rRNA gene is a reliable phylogenetic marker but is not an effective identification tool as it does not allow discrimination among closely related species. A multilocus sequence analysis (MLSA), on the contrary, effectively offers a high resolution and yields a robust identification system. MLSA compares the primary DNA sequences from multiple conserved protein coding loci for assessing the diversity and relation of different isolates across related taxa. The combined use of the phenylalanyl-tRNA synthase alpha subunit (*pheS*) and RNA polymerase alpha subunit (*rpoA*) gene sequences offers a reliable identification system for nearly all *Lactobacillus* species (Naser, 2006).

2. LITERATURE REVIEW

2.1. The genus *Lactobacillus*

Members of the genus *Lactobacillus* are not only found in plants and in plant-derived materials, such as silage, grains and foods, but also in the gastrointestinal tract (GIT) of humans and animals (Stewart, 1997). Species of *Lactobacillus* form the most numerous genus in the heterogeneous group of LAB. Lactobacilli are Gram-positive, non-sporeforming and strictly fermentative bacteria producing lactic acid as the primary end product (Salminen and von Wright, 1998; Makarova et al., 2006). The lactobacilli have been formely classified into three physiological groups on the basis of their carbohydrate metabolism i.e. (1) the obligatory homofermantative, which possess fructose-1,6-biphosphate aldose and ferment hexoses, (2) the facultative heterofermantative lactobacilli, which possess both aldose and phosphoketolase and ferment hexoses, pentoses and gluconate and (3) the obligatory heterofermantative species lacking aldolase (Hammes and Vogel, 1995).

Approximately one hundred species described for the genus *Lactobacillus* (http://www.bacterio.cict.fr/l/lactobacillus.html) are subdivided by 16S rRNA analysis, DNA-DNA hybridization and other phylogenetic methods, into eight major groups; *Lactobacillus buchneri*, *Lactobacillus delbrueckii*, *Lactobacillus casei, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus sakei, Lactobacillus salivarius,* and *Lactobacillus brevis* group (Salminen and von Wright, 1998; Dellaglio and Felis, 2005). The *L. delbrueckii* group was later renamed the *Lactobacillus acidophilus* group. Based on DNA-DNA-hybridization studies, the large *L. acidophilus* group is divided into six groups, A1-A4 and B1-B2, that correspond to previously assigned species, *L. acidophilus* (A1), *L. crispatus* (A2), *L. amylovorus* (A3), *L. gallinarum* (A4), *L. gasseri* (B1) and *L. johnsonii* (B2) (Fujisawa et al., 1992). Although six genome clusters of the *L. acidophilus* group have been designated as separate species with validly published names, they are difficult to distinguish solely on the basis of phenotypic characteristics. Recent *Eco*RI ribotyping data further suggests that the group should be divided into 14 genotypes, A1-A11, B1-B3, and gives evidence that some of the previously identified

Lactobacillus strains would require reclassification as different species (Ryu et al., 2001). The taxonomy of the genus *Lactobacillus* has changed considerably as a consequence of the introduction of new genomic techniques for the identification of *Lactobacillus*.

The heterogeneity poses challenges and opportunities when characterizing or exploiting individual strains. At the time of writing (August 2007), 11 *Lactobacillus* genome sequences have been published, and at least 12 more sequencing projects are ongoing (Makarova and Koonin, 2007). These studies will dramatically improve our understanding of metabolic processes, bioprocessing capabilities and potential roles of the lactobacilli in health and well-being.

2.2. *Lactobacillus* in the normal intestinal microbiota

The normal intestinal microbiota works as a barrier against pathogens, contributes to degradation of some food components, stimulates the host immune system, and produces certain vitamins, enzymes and short-chain fatty acids (Holzapfel et al., 1998). The normal gut bacterial population of an adult human is estimated to comprise more than 400 species, with the predominance of obligate anaerobes (Rolfe, 1997). The presence and composition lactobacilli in the microbiota of the GIT of mammalian animals closely resemble those found in humans, although some variations at the species level occur depending on the host (Tannock, 1999). Also, the anatomical differences of the alimentary canals influence the microbiota: e.g. the non-secreting stratified squamous epithelia in the fore-stomach of pigs are efficiently colonized by lactobacilli (Tannock, 1997).

2.2.1. *Lactobacillus* in porcine gastrointestinal tract

The porcine GIT harbours an extremely complex microbiota which has a profound impact on host's health. Several studies have investigated the species diversity of the pig intestine through phenotypic analysis of isolates obtained by anaerobic culturing (Tannock and Smith, 1970; Salanitro et al., 1977; Robinson et al., 1981; Varel, 1987). Culturing, however, is likely to recover some bacteria more readily than others, and it is laborious to perform (Zoetendal et al., 2004). The development of new molecular tools has revolutionized our knowledge of gut microbial diversity (Pryde et al., 1999; Leser et al., 2002). Unlike in humans, lactobacilli are the dominant LAB in the pig intestine (at the level of 9 log bacterial cells per gram chyme) (Konstantinov et al., 2004).

Lactobacilli establish early in the piglet's intestine, and although succession occurs throughout the pig's lifetime, they remain a predominant part of the intestinal bacterial community. Several reports showed that three major groups of *Lactobacillus* spp. are identified from the GIT of pigs. (1) The first group comprises obligately homofermentative lactobacilli, typically represented by members of the *L. acidophilus* group such as *L. amylovorus*, *L. crispatus*, *L. gallinarum*, and *L. acidophilus*. (2) The second group of lactobacilli associated with the GIT of pigs comprises facultatively heterofermentative strains. The only species of this group that has been identified frequently is *L. plantarum*. (3) The third group includes a rather large number of obligately heterofermentative species including *L. reuteri*, *L. mucosae*, and *L. rossiae*. *L. reuteri* –related strains were shown to constitute the major part of the heterofermentative lactobacilli identified from pig faeces (Pryde et al., 1999).

2.2.2. *Lactobacillus* as probiotics

The findings that colonization by lactobacilli and other lactic acid bacteria, improves infection resistance of the host, have led to the production and consumption of probiotics. A probiotic organism is a live microbial supplement that beneficially affects

the health and nutrition of the host (Salminen et al., 1996). To date, several health-promoting effects of probiotics have been proposed e.g. prevention of the pathogen colonization in the GIT via competitive exclusion (CE), and/or synthesis of inhibitory compounds (Kaur et al., 2002; Isolauri et al., 2004). The ability to adhere to intestinal mucosa is considered an important requirement for microorganisms intended for probiotic use, allowing at least a temporary colonization of the human and animal intestinal tract. As microbial feed additives, they offer potential as an alternative to antimicrobials; both as a means of controlling pathogen carriage and improving growth rate and feed conversion. However, the mechanisms underlying the health effects and the host-probiotic communication in prophylactic and/or therapeutic treatments have remained poorly characterized. The probiotic strains are expected to fulfil several health-promoting characteristics and safety criteria. These are listed in Table 1.

Table 1. Expected characteristics and safety criteria of probiotics (Mercenier et al., 2003).

Non toxic and non pathogenic
Accurate taxonomic identification
Normal inhabitant of the targeted species
Capability to survive, proliferate and be metabolically active in the targeted site, which implies:
resistance to gastric juice and bile
ability to persistent in the GIT
ability to adhere
ability to compete with the resident microbiota
Production of antimicrobial substances
Antagonism towards pathogenic bacteria
Ability to modulate immune responses
Ability to exert at least one clinically documented health benefit
Genetically stable
Amenability of the strain and stability of the desired characteristics during the processing, storage and delivery
Viability at high populations
Desirable organoleptic and technological properties when included in industrial prosesses
Isolation from suitable habitats

2.2.3. Adhesive properties in *Lactobacillus*

For successful colonization, intestinal bacteria, including lactobacilli, have been suggested to resist the peristaltic movement by adhering to intestinal epithelia and/or mucus, particularly in the upper parts of the alimentary canal (Rojas and Conway, 1996). Several factors contribute to the interaction of lactobacilli with the host tissues, such as cell surface hydrophobicity and autoaggregation (Kos et al., 2003), lipoteichoic acids (Granato et al., 1999) and cell surface proteins.

Lactobacilli have been frequently observed to bind to epithelial cells and dissected tissue samples of the alimentary canal from human and animals (Fuller, 1973; Kotarski and

Savage, 1979; Mäyrä-Mäkinen et al., 1983; Conway et al., 1987; Conway and Adams, 1989; Henriksson et al., 1991; Sarem-Damerdji et al., 1995; Jin et al., 1996; Yuki et al., 2000), to intestinal mucus (Rojas and Conway, 1996; Kirjavainen et al., 1998; Kirjavainen et al., 1999; Matsumura et al., 1999; Tuomola et al., 1999; Roos et al., 2000; Roos and Jonsson, 2002; Gusils et al., 2003; Collado et al., 2007), to cultured human carcinomal intestinal cell lines (Adlerberth et al., 1996; Granato et al., 1999; Kirjavainen et al., 1999) and to the components of the extracellular matrix (ECM) (Nagy et al., 1992; Harty et al., 1994; McGrady et al., 1995; Toba et al., 1995; Styriak et al., 2003). The level of adhesion has been estimated either by microscopic visualization or by using metabolically radiolabelled bacteria.

Reports on the adherence are numerous, but detailed knowledge of the adhesion mechanisms is very limited. Species-specificity in the adherence of lactobacilli has also been suggested (Fuller, 1973; Mäyrä-Mäkinen et al., 1983; Yuki et al., 2000), but the topic has remained controversial, since intestinal and environmental lactobacilli adhere to non-host tissue targets as well (Kotarski and Savage, 1979; Conway et al., 1987; Sarem-Damerdji et al., 1995; Jacobsen et al., 1999). In general, lactobacilli seem to express preference for adhesion to epithelial cells of their own host but a strict host-species specifity of the adhesion seems unlikely.

Caco-2 and Intestine 407 cell lines, ECM proteins and mucus have been commonly used in adhesion studies with lactobacilli. The Caco-2 cell layer isolated from a human colonic adenocarcinoma (Fogh et al., 1979) structurally resembles differenciated enterocytes at the intestinal epithelium (Pinto et al., 1983). Intestine 407 cell line derived from a malignant small intestine of a human embryo (Henle and Deinhardt, 1957) does not differentiate to a polarized cell layer (Favre-Bonte et al., 1995). The adhesion mechanisms involved are not known, in one report mannosyl conjugates were proposed as adhesion targets (Adlerberth et al., 1996).

ECM proteins are distributed in basal membrane (BM) and the intestinal matrix, which underlie the intestinal epithelial cells (Fig. 1). They may become exposed by trauma, or viral and bacterial infections (Ljungh and Wadstrom, 1996). Furthermore, the normal

shedding of epithelia through cell turnover provides the epithelial surface with ECM components. The ECM is involved in cellular development and function, growth and differentation, cell adhesion as well as migration. The main components of ECM belong to three major classes of biomolecules such as 1) structural proteins: collagen and elastin, 2) specialized proteins: e.g. fibrillin, fibronectin and laminin, and 3) proteoglygans. ECM proteins exposed on the epithelia are used as targets for bacterial adhesion. The adherence of pathogens to ECM components has been investigated thoroughly and shown to promote colonization and virulence of the pathogens (Westerlund and Korhonen, 1993). To prevent intestinal infection, it is therefore important to prevent the adherence of pathogenic bacteria to ECM proteins, as well as to epithelial cells. Such a *Lactobacillus* adherence may protect the host against bacterial invasion at damaged epithelia where the ECM has become exposed.

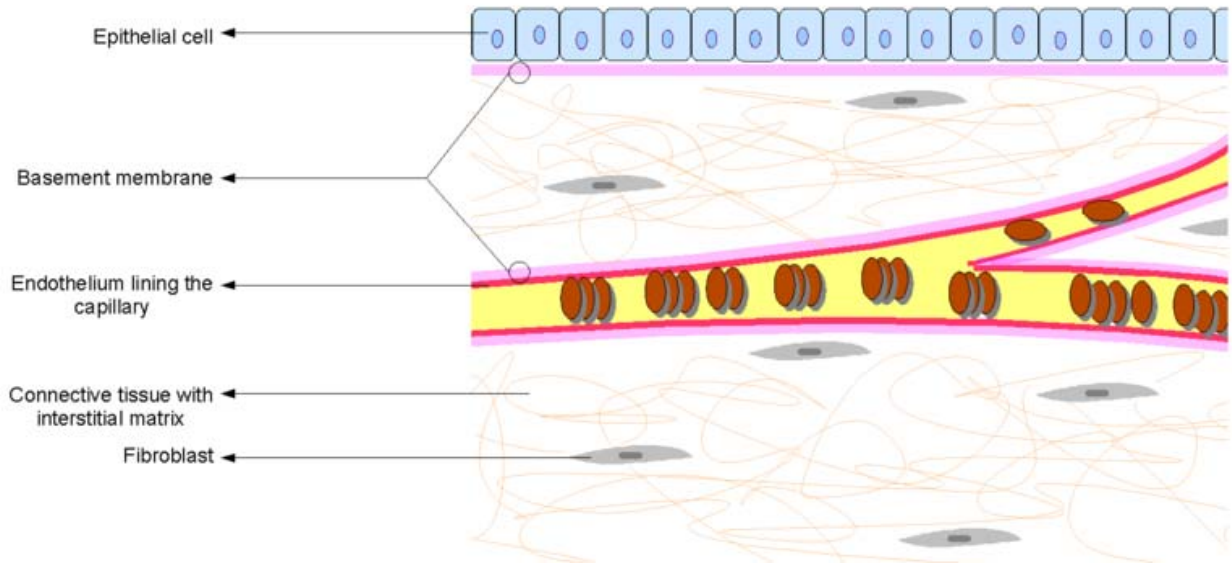

Fig.1. Illustration depicting ECM in relation to epithelium, endothelium and connective tissue adapted from Wikipedia September 2007 (http://en.wikipedia.org/wiki/Extracellular_matrix).

There are number of studies that report binding of lactobacilli strains to mucus from animals (Roos et al., 2000; Rojas et al., 2002; Gusils et al., 2003; Sun et al., 2007). As the outermost luminal layer, mucus is the first intestinal component or surface that lactobacilli are likely to contact before reaching the epithelial cells. Hence, this binding to mucus can have a substantial role in the colonization of intestinal surfaces. The main structural components of mucus are large molecules called mucins. Mucins provide physical protection to epithelia and facilitate the smooth transit of ingested food material (Tannock, 1999).

The reduced adhesiveness of lactobacilli treated with proteinases has led to the hypothesis that proteinaceous molecules mediate the adhesion of lactobacilli in the host intestine (Fuller, 1975; Henriksson et al., 1991; Reid et al., 1993; Greene and Klaenhammer, 1994). These include the mucus binding proteins from *L. reuteri* (Roos and Jonsson, 2002; Bath et al., 2005), *L. acidophilus* (Buck et al., 2005) and *L. plantarum* (Boekhorst et al., 2006). In addition to the high molecular weight (MW) mucus binding protein of *L. reuteri* (Roos and Jonsson, 2002), mucus/collagen binding proteins with sequence similarity to solute binding proteins of ABC transporters have been described for *L. reuteri* strains (Miyoshi et al., 2006). Furthermore, genome analysis of *L. plantarum* has revealed several cell surface proteins with adhesive domain structures (Boekhorst et al., 2006). The involvement of carbohydrates and lipoteichoic acids in the adherence of lactobacilli to intestinal epithelia has also been reported (Fuller, 1975; Henriksson et al., 1991; Coconnier et al., 1992; Greene and Klaenhammer, 1994; Adlerberth et al., 1996; Ahrne et al., 1998; Boris et al., 1998; Granato et al., 1999; Neeser et al., 2000), but the adhesive receptors have not been identified. Overall, the various results suggest that lactobacilli adhere to host tissues via mechanisms that vary in different species.

A few adhesins of lactobacilli have been characterized at the molecular level. These include the collagen binding CnBP of *L. reuteri* (Aleljung et al., 1994; Rojas and Conway, 1996), the collagen and laminin-binding CbsA of *L. crispatus* (Sillanpää et al., 2000; Antikainen et al., 2002), fibronectin binding SlpA of *L. brevis* (Hynönen et al., 2002), and

the pig and hen mucus-binding Mub and MapA of *L. reuteri* (Roos and Jonsson, 2002; Miyoshi et al., 2006).

2.3. S-layer proteins (Slps) of *Lactobacillus*

S-layers are crystalline arrays of proteinaceous subunits located at the outermost part of the cell wall in several species of the genus *Lactobacillus*, as well as in many other bacteria and *Archaea*. Lactobacillar S-layers are relatively small, 25 kDa to 71 kDa in size (Åvall-Jääskeläinen and Palva, 2005), whereas the molecular masses of S-layers in other bacterial species range up to 200 kDa (Sara and Sleytr, 2000). S-layers are normally 5–15 nm thick possessing a smoother outer surface compared with a more structured inner surface. Each S-layer forms a highly porous structure with pores of an identical size and morphology. Based on electron microscopy, the S-layer subunits are composed of lattices with oblique, square or hexagonal symmetry (Sara and Sleytr, 2000). The oblique lattice type was identified in the S-layers of *L. acidophilus* (Smit et al., 2001), *L. brevis* (Jakava-Viljanen et al., 2002) and *L. helveticus* (Lortal et al., 1997) and the hexagonal lattice type in *L. casei* and *L. buchneri* (Masuda and Kawata, 1985). The S-layer subunits are non-covalently linked to each other and to the supporting cell envelope, and can be disintegrated into monomers by denaturing agents such as urea or GHCl, by metal-chelating agents or by cation substitution (Sara, 2001). In addition to peptidoglycan, the rigid cell envelope of lactobacilli is composed of secondary cell wall polymers (SCWP) such as teichoic acid, lipoteichoic acids, lipoglycans or neutral or acidic glygans (Navarre and Schneewind, 1999; Neuhaus and Baddiley, 2003).

S-layer protein encoding genes have been cloned and sequenced from two *L. brevis* strains (Vidgrén et al., 1992; Jakava-Viljanen et al., 2002), two *L. acidophilus* strains (Boot et al., 1993; Altermann et al., 2005), one *L. helveticus* strain (Callegari et al., 1998), one *L. crispatus* strain (Sillanpää et al., 2000) and seven *L. gallinarum* strains (Hagen et al., 2005). Deposited in GeneBank (National Center for Biotechnology Information, Bethesda, MD, USA) are also several S-layer protein encoding gene

sequences, which are either unpublished or have not been described in detail in publications. Additionally, strains of *L. amylovorus, L. buchneri, L. kefir* and *L. parakefir* have also been shown to possess an S-layer (Boot et al., 1996b; Garrote et al., 2004), but their S-layer protein genes have not yet been sequenced. The presence of multiple S-layer protein genes seems to be quite common for lactobacilli. Formerly, *L. johnsonii* and *L. gasseri* were proposed to lack an S-layer (Boot et al., 1996), but Ventura et al. (2002) identified the protein called aggregation-promoting factor from these species as an S-layer-like protein, having amino acid composition and physical properties similar to lactobacillar S-layers. However, the formation of a regular lattice structure has not been demonstrated. Several different strains of these species were shown to possess two genes encoding surface proteins, one silent and one actively transcribed (Boot et al., 1995; Sillanpää et al., 2000). These bacteria may express alternative S-layer protein genes, most likely to adapt to different stress factors such as drastic changes in the environmental conditions. In most of the characterized cases, the mechanism of S-layer variation is based on DNA rearrangements, but in *L. brevis* ATCC 14869, the variation of S-layer protein content took place by a unique mechanism involving activation of transcription by a soluble factor as a result of an environmental change (Jakava-Viljanen et al., 2002).

As S-layer proteins represent 10-15% of the total amount of proteins in *Lactobacillus* cells (Boot and Pouwels, 1996), their transcription and secretion mechanisms must be efficient and tightly regulated. Multiple promoters precede several S-layer genes (Boot and Pouwels, 1996), including S-layer genes of *L. acidophilus* (Boot et al., 1996) and *L. brevis* (Vidgrén et al., 1992; Kahala et al., 1997; Jakava-Viljanen et al., 2002) and are likely to ensure efficient transcription of these genes.

Despite their similar amino acid composition, such as a low content of cysteine and methionine as well as a high content of hydrophobic amino acids and hydroxyl amino acids, sequence similarity between the S-layer protein genes can only be found between genes of related species (Åvall-Jääskeläinen and Palva, 2005). This has also been demonstrated for the S-layer protein genes of lactobacilli by DNA–DNA hybridizations.

The gene and protein sequences of 10 S-layer proteins belonging to group A of the *L. acidophilus* complex are currently available in GeneBank (Hagen et al., 2005). A comparison of the amino acid sequences of these proteins has revealed two conserved regions, namely, an N-terminal signal sequence of ca. 30 amino acids, which directs secretion of the protein by the general secretory pathway and a C-terminal domain of approximately 123 amino acids that anchors the protein to the cell surface. A variable domain is located between the two conserved regions.

The functions of Slps are not yet completely revealed but it has been proposed that these structures protect the microbe from hostile environmental agents and aid in maintaining cellular integrity (Åvall-Jääskeläinen and Palva, 2005). Several lactobacillar S-layers have been identified as putative adhesins with an affinity for various tissue compartments or molecules. Slps of lactobacilli have been shown to confer tissue adherence, including *L. crispatus* and *L. acidophilus,* whose ability to bind to host epithelial cells is decreased after removal or disruption of the S-layer proteins (Sillanpää et al., 2000; Buck et al., 2005; Frece et al., 2005). The *L*. *brevis* ATCC 8287 SlpA protein has been shown to possess affinity for human intestinal epithelial cell lines, urinary bladder, endothelial cells and fibronectin (Hynönen et al., 2002). The ability of the receptor-binding region of SlpA to adhere to fibronectin was also confirmed with a lactococcal surface display system (Åvall-Jääskeläinen et al., 2003). Recently, by using surface plasmon resonance, SlpA was found to interact with fibronectin and laminin whereas its interaction with collagen and fibrinogen was found to be of much lower affinity (de Leeuw et al., 2006). In addition, S-layer protein extracts from *L. helveticus* have been shown to inhibit enterohaemorrhagic *E. coli* adhesion to host epithelial cells (Johnson-Henry et al., 2007).

2.4. F18$^+$ *E. coli*

Newly weaned piglets are highly susceptible to F18$^+$ enterotoxigenic *E. coli* (ETEC) and F18$^+$ verotoxigenic *E. coli* (VTEC) infections. F18$^+$ *E. coli* infections causing PWD and

ED in young pigs occur mostly 1–2 weeks post-weaning (Bertschinger et al., 1990) and lead to considerable economic losses in pig farms. $F18^+$ *E. coli* strains adhere to the microvillus of the small intestinal epithelial cells of weaned pigs but not to newborn pigs (Nagy, B. et al., 1992). F18 fimbriae are an important virulence factor of ETEC and VTEC. Adherence of $F18^+$ *E. coli* to porcine intestinal epithelial cells is mediated by the adhesin (FedF) of F18 fimbriae. Antibodies against FedF were seen to inhibit the adhesion of $F18^+$ *E. coli* to porcine enterocytes (Smeds et al., 2001; Smeds et al., 2003). Susceptibility to these $F18^+$ *E. coli* infections in pigs is shown to be dependent on the presence of the specific F18 receptor (F18R) on the porcine intestinal epithelial cells (Bertschinger et al., 1993; Frydendahl et al., 2003). Binding of F18 fimbriae to the F18R on the brush border of porcine enterocytes result in colonization of the small intestine. This leads to subsequent secretion of entero- or verotoxins.

Pig populations consist of F18R negative ($F18R^-$) and F18R positive ($F18R^+$) animals and only the latter are subject to infection with $F18^+$ *E. coli* (Frydendahl et al., 2003). The F18R status of pigs is genetically determined (Bertschinger et al., 1993) and susceptibility to $F18^+$ *E. coli* infections appeared to be dependent on the activity of the *FUT1* gene, encoding the alpha (1,2)-fucosyltransferase (FUT1) (Meijerink et al., 1997; Meijerink et al., 2000). Despite the presence of the F18R, there is a low prevalence of $F18^+$ *E. coli* infections in suckling piglets. This suggests the importance of inhibiting the molecules present in sow's milk. Indeed, it has been shown that antibodies in sow's milk provide protection to piglets against enteropathogens such as $F4^+$ *E. coli* (Riising et al., 2005).

The *fed* gene cluster is composed of five genes (Fig. 2). The gene encoding the major protein, FedA, and two genes encoding the minor proteins, FedE and FedF, were first described by Imbrechts et al. (1992, 1996). The rest of the *fed* genes, *fedB* and *fedC*, were characterized and found to encode the putative usher protein (FedB) and chaperone (FedC) of F18 fimbriae. Ushers are large (80-100 kDa) outer membrane proteins found in most *E. coli* fimbrial determinants. In fimbrial biosynthesis, fimbrial subunits are translocated to extracellular sites through a channel made up of several

usher proteins. Chaperones cap the subunits of the fimbrial complex and protect them from premature assembly and proteolysis. The specific adhesive property of F18 is associated with FedF, but the function of FedE has remained unknown (Smeds et al., 2001).

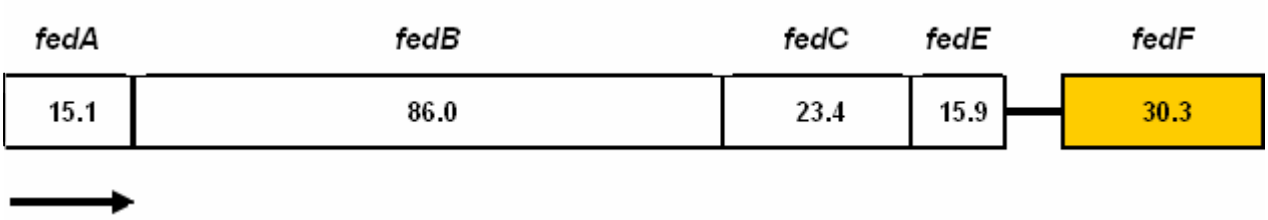

Fig. 2. Genetic organization of the *fed* gene cluster adapted from Smeds et al. (2001). Molecular masses calculated for the gene products are given in kDa in the boxes. The encoding the adhesion of F18 fimbriae is presented by the yellow box.

There are two variants of F18 fimbriae, differing in the amino acid sequence of the major fimbrial subunit FedA (Nagy et al., 1997). This variation is responsible for the antigenic difference between F18ab and F18ac, the latter containing the FedA with an extra proline residue (Imberechts et al., 1994; Rippinger et al., 1995; Verdonck et al., 2004). The F18ac variant is mostly produced by ETEC strains, whereas F18ab by VTEC strains (Wittig et al., 1994; Nagy et al., 1997; Bosworth et al., 1998). The variants can be distinguished by serology and by restriction enzyme digestion of PCR products (Bosworth et al., 1998). The F18ab variant is often expressed by VTEC O139 strains and causing porcine edema disease (ED). The F18ac fimbrial *E. coli* strains often belong to serogroup O141 or O157 and cause diarrhoea by expressing enterotoxins (STa or STb) either together with or without verotoxin (Nagy et al., 1997).

Typical signs of ED in pigs are subcutaneous edema of the eyelids, neurological signs and sometimes also diarrhoea. After onset of signs, ED is fatal even when treated with antimicrobials and pigs often die without previous signs of illness. Morbidity is usually

low, but as case fatality can reach 90%, some farms may suffer from mortality rate of up to 60%. The signs of PWD in pigs include watery diarrhoea or sometimes a mixture of ED and PWD. These infectious endemic porcine diseases are widely spread (Verdonck et al., 2003; Cheng et al., 2005) and are a major cause of economic losses in the pig industry due to diarrhoea, growth retardation and mortality. PWD caused by $F18^+$ *E. coli* is also present in Finland.

Several methods to prevent $F18^+$ *E. coli* infections have been examined (Imberechts et al., 1997; Kyriakis et al., 1997; Nollet et al., 1999; Felder et al., 2000; Tsiloyiannis et al., 2001), but no vaccine or effective therapy exist to date. If F18 fimbriae-specific antibodies are present due to active immunization, reduction of $F18^+$ *E. coli* colonization has been reported (Sarrazin and Bertschinger, 1997; Bertschinger et al., 2000). However, mucosal immunization of piglets with purified F18 fimbriae does not protect them against $F18^+$ *E. coli* infection (Verdonck et al., 2007).

Because the FedF adhesin is only a minor component of the F18 fimbriae, whole fimbriae do not usually obtain a strong antibody response to the adhesin or protection against the $F18^+$ *E. coli* infection. Oral immunization of piglets with purified F4 fimbriae was reported to induce a protective F4-specific immune response (Van den Broeck et al., 1999). However, the adhesive subunit of F4 fimbriae is the major subunit FaeG. The F18 fimbrial adhesin FedF is conserved among $F18^+$ *E. coli* field isolates from different places around the world (Tiels et al., 2005). Therefore, recombinant FedF or its lectin domain (Smeds et al., 2003) should be considered for oral immunization of piglets.

2.5. *Lactobacillus* as live vaccine delivery vectors

Lactobacilli possess a number of properties which make them attractive candidates for oral vaccination purposes. These bacteria are considered to be safe organisms with a GRAS (generally regarded as safe) status. This is in contrast to other live vaccine carriers used (e.g., *Salmonella*, *E. coli*, vaccinia virus), which cannot be classified as safe. Furthermore, a particularly attractive feature of lactobacilli is their capacity to

colonize certain regions of the mucosa, which permits induction of local immune responses. An additional benefit of the use of lactobacilli is that some strains are considered to show health-promoting (probiotic) activities for humans and animals (Pouwels et al., 2001).

Oral presentation of antigens offers a number of advantages over other routes (parenteral administration). Oral administration of vaccines is convenient, can be carried out on a large scale and is relatively inexpensive. Furthermore, many pathogens enter the body via the mucosal surfaces of the body. Moreover, oral immunization frequently evokes both local and systemic immune responses, resulting in an effective elimination of foreign invaders (Mercenier et al., 2000).

S-layers have been identified in several *Lactobacillus* species. In some of these bacteria, S-layers have been shown to function as adhesins mediating binding of *Lactobacillus* cells to the host's epithelial cells and/or ECM (reviewed in Åvall-Jääskeläinen and Palva, 2005). Due to these observed adhesive properties, including their high degree of structural regularity and their self-assembly properties, the possible therapeutic applications of lactobacillar S-layers have gained increasing interest e.g. as targeted live antigen delivery vehicles to host tissues (Shaw et al., 2000; Reveneau et al., 2002; Scheppler et al., 2002). S-layer could provide a superior expression level and surface density of the required antigen as compared to other bacterial antigen presentation systems. It has already been demonstrated that S-layer protein subunits can be modified to carry foreign epitopes as a uniform recombinant S-layer on the *Lactobacillus* cell surface (Smit et al., 2002; Åvall-Jääskeläinen et al., 2002). Immunization studies with *Lactobacillus* S-layer antigen constructs are still lacking, but the results of the few immunization studies utilizing recombinant S-layer proteins of other bacteria (Umelo-Njaka et al., 2001; Riedmann et al., 2003) further encourage the development of *Lactobacillus* S-layer based antigen vectors. Immunization studies with piglets need be conducted with *Lactobacillus* vaccine vectors surface-displaying the receptor-binding domain of *E. coli* F18 fimbriae as part of the S-layer before conclusion on the suitability of these contructs as live vaccine vectors can be drawn.

3. AIMS OF THE STUDY

This study was aimed to isolate new S-layer positive *Lactobacillus* species from the porcine intestine and faeces for the later use as vaccine vectors and/or probiotics for pigs, to identifiy the lactobacilli found by using polyphasic taxonomic studies and to test S-layer-expressing strains for their ability to adhere to different host tissues. The S-layer of *L. brevis* ATCC 14869 was chosen for basic characterization to aid structure-function analysis.

This study was part of a larger project aimed at developing a live oral LAB vaccine against PWD and ED in pigs caused by $F18^+$ *E. coli.*

The specific aims were:

1. To isolate S-layer proteins carrying *Lactobacillus* species from the pig intestine and faeces in order to obtain host specific strains.
2. To identify and characterize the porcine-specific S-layer proteins carrying *Lactobacillus* strains.
3. To characterize the genes encoding the S-layer protein of *L. brevis* ATCC 14869 and study their expression in different growth conditions.
4. To develop adhesion assays to test S-layer-expressing strains and the adhesin of *E. coli* F18 fimbriae for their ability to adhere to host tissues.
5. To characterize the adhesin of *E. coli* F18 fimbriae and further test the adhesin on the intestinal epithelium of piglets.

4. MATERIALS AND METHODS

The bacterial strains, plasmids and cell line used in this study are listed in Table 2.The methods are described in detail in the original articles and summarized in Table 3.

Table 2. Bacterial strains, plasmids and cell line used in this study.

Bacterial strain, plasmid and cell line	Origin/relevant property	Article	Reference and/or source
Bacterial strains			
L. amylovorus DSM 20531 type	cattle waste-corn fermentation	II	Nakamura, 1981, DSMZ
L. amylovorus DSM 20532	cattle waste-corn fermentation	II	Nakamura, 1981, DSMZ
L. amylovorus DSM 20552	intestine of adult	II	Lauer et al., 1980, DSMZ
L. amylovorus LMG 9496 type	cattle waste-corn fermentation	II	Nakamura, 1981, LMG
L. sobrius DSM 16698 type	piglet faeces	II	Konstantinov et al., 2006, DSMZ
L. sobrius AD5	piglet faeces	II	Konstantinov et al., 2006
L. amylovorus LAB2	porcine faeces	I, II	These studies
L. amylovorus LAB7	porcine faeces	I, II	These studies
L. amylovorus LAB8	porcine faeces	I, II	These studies
L. amylovorus LAB13	porcine ileum	I, II	These studies
L. amylovorus LAB16	porcine jejunum	I, II	These studies
L. amylovorus LAB31	porcine jejunum	I	This study
L. amylovorus LAB52	porcine ileum	I, II	These studies
L. crispatus LAB32	porcine jejunum	I	This study
L. mucosae LAB87	porcine ileum	I	This study
L. brevis ATCC 8287	green, fermenting Sevillano variety olives	I	(Orla-Jensen) Bergey et al., 1934, ATCC
L. brevis ATCC 14869 type	human faeces	III	Orla-Jensen, 1919, ATCC
E. coli 107/86	edema disease strain	IV	Bertschinger et al., 1990
E. coli HB101	host for pIH120 vector	IV	Boyer and Roulland-Dussoix, 1969
Plasmids			
pIH120	F18 fimbriae expression vector	IV	Imbrecht et al., 1996
pMAL-p2	expression vector for MBP fusions	IV	New England Biolabs
pKTH2095	LAB cloning vector	III	Savijoki et al., 1997
Cell line			
Intestine 407 cell line	Adhesion test	I	Henle and Deinhardt, 1957, ATCC

ATCC, American Type Culture Collection
DSMZ, Deutsche Sammlung von Microorganismen und Zellkulturen
LMG, Belgian Co-ordinated Collections of Microorganisms

Table 3. Methods used in this study.

Method	Described and used in
Morphology and phenotypical tests	I, II
DNA methods and transformation	
Isolation of chromosal DNA	I, II, III, IV
Polymerase chain reaction (PCR)	I, II, III, IV
Real-time quantitative PCR	III
Vectorette system	III
Dot blot hybridization	I
Southern blotting and hybridizatiton	III
DNA sequencing and sequence analyses	I,II,III,IV
Ribotyping	II
DNA-DNA reassociation	II
PCR-ELISA	I
Transformation of *L. brevis*	III
Transformation of *E. coli*	IV
RNA methods and RT-PCR	
RNA isolation	III
RNA gel electrophoresis and Northern blotting	III
Primer extension studies and RT-PCR	III
Adhesion assays	
In vitro adhesion to porcine epithelial cells	I, IV
Bacterial adherence to a cell line	I
Bacterial adherence to the extracellular matrix (ECM)	I
Immunological methods	
Indirect immunofluorescence (IFA)	IV
Protein and enzyme assays	
SDS-PAGE	I, III, IV
Immunoblot analyses	III
Peptide sequencing and mass mapping	III, IV
Aminopeptidase activity assays	III
Extraction of S-layer proteins	I
Electron microscopy	III
Affinity chromatography	IV
Production of antisera	III, IV

5. RESULTS AND DISCUSSION

5.1. Isolation and identification of porcine-specific S-layer carrying *Lactobacillus* strains (I)

Lactobacillus strains, carrying a putative S-layer or an *L. brevis slpA* homolog, were isolated from the intestine and faeces of pigs using biochemical and genetic screening for later use as vaccine vectors and/or probiotics for pigs.

In total 99 strains, putatively belonging to the genus *Lactobacillus*, were isolated from the small intestine (duodenum, jejunum and ileum) of four newly slaughtered pigs and two sow's faeces as pure culture. The identification at the genus level was made in this study by using a PCR-ELISA assay developed and verified earlier for the analysis of the genus *Lactobacillus* (Laitinen et al., 2002). SDS-PAGE and a gene probe specific for the *L. brevis* ATCC 8287 S-layer protein gene (*slpA*) were used to screen for the presence of strains possessing putative surface-layer proteins (Slps).

The identification, based on phenotyping and 16S rRNA sequences, revealed that the eight S-layer protein producing strains were closely related to *L. amylovorus* and *L. crispatus*. The presence of a putative S-layer on the bacterial cell surface can be deduced from the incidence of a dominant band in the protein profile of non-lysed bacteria. In this study, dominant protein bands of 45-62 kDa were present in 8 of 99 strains (Fig. 1 in I), suggesting the presence of S-layer proteins in these strains. After 16S rRNA based species identification, the presence of genes encoding Slps could be confirmed by PCR. The PCR products were partially sequenced to reveal homology in the conserved C-terminal domain encoding part in this type of S-layer genes. A sequence analysis of a conserved C-terminal fragment indicated that all SDS-PAGE positive strains contain genes homologous to the *slp* genes of related *Lactobacillus* strains.

To test whether the genomes of the 99 isolates shared DNA identity with the *slp* gene of *L. brevis* ATCC 8287, chromosomal DNA was isolated from the strains followed by

hybridization with an *slpA* probe. Only one of the isolates gave a positive hybridization signal, but no *L. brevis* –like strains could be isolated. The only strain found, with no apparent S-layer protein, was identified as *L. mucosae*, which is not phylogenetically related to *L. brevis*. It is likely that the observed homology is not linked to a silent *slp* gene but rather to conserved cell wall binding domains of wall-associated proteins.

5.2. Adhesion properties of Slps of lactobacilli to different host tissues (I)

In this study, the ability of putative S-layer-expressing strains to adhere to pig and human intestinal cells was tested. We found that the putative S-layer-protein-expressing cells of porcine isolates adhered very efficiently to pig enterocytes. Adhesive strains were attached on the epithelial cells, whereas non-adhesive strains had a uniform distribution around the cells. It was noteworthy that some porcine isolates also adhered to human cells with a high affinity, indicating that the adhesiveness of lactobacilli is not strictly host-specific.

ECM proteins have also been used as a model of damaged intestinal mucosa. To investigate the role of the putative S-layer in the ECM binding, adhesion of S-layer-expressing and S-layer-depleted bacteria to human fibronectin, human laminin, and mouse type IV collagen were also examined. Strong adhesion to fibronectin was observed in the presence of the S-layer proteins in *L. brevis* ATCC 8287 and six out of the eight studied porcine *Lactobacillus* isolates, whereas the removal of the intact Slps from the bacterial surface by extraction with guanide hydrochloride (GHCl) abolished the adhesiveness of these bacteria. Adhesion to laminin was observed in seven S-layer carrying lactobacilli strains. Interestingly, the adhesiveness to laminin increased in three strains after abolishment of the S-layer structure. With exception of one strain, no adherence to type IV collagen was observed irrespective of the GHCl treatment. In the exceptional strain, collagen binding was also independent of the GHCl treatment, indicating that the adhesion was not mediated by the putative S-layer. The strain

showing no presence of S-layer subunits but hybridising with *L. brevis slpA,* did not exhibit any detectable adhesiveness in the binding tests performed.

Extraction with GHCl, as used in this study, is a routine procedure to remove S-layer proteins and did not cause detectable lysis of the cells (Sara, 2001). However, it is obvious that some other cell wall proteins or components, critical to binding activity, may be at the same time removed. Thus, it is clear that surface structures other than the S-layer protein mediate adhesion in such strains, e.g. lipoteichoic acids have been shown to contribute to the adhesion process (Antikainen et al., 2002). The poor adhesiveness of S-layer-depleted strains to fibronectin and laminin is, however, in accordance with the observation that S-layer subunits indeed are adhesion proteins and one of the key adhesion factors of the cell.

5.3. Polyphasic taxonomic studies of *Lactobacillus* strains (II)

While studying the taxonomy of six *Lactobacillus* isolates from porcine intestine and faeces, the taxonomy position of *Lactobacillus sobrius* type strain DSM 16698^T and AD5 strain based on comparative 16S rRNA sequence analysis was found controversial, as they showed a high similarity to the *Lactobacillus amylovorus* species. Therefore, the taxonomy of these species was addressed in a polyphasic taxonomy study that including, in addition to re-evaluating the 16S rRNA gene sequence and DNA-DNA reassociation results (Supplementary Table S1 in II), multilocus sequence analysis (MLSA) of housekeeping genes encoding the phenylalanyl-tRNA synthase alpha subunit (*pheS*) and RNA polymerase alpha subunit (*rpoA*) (Fig. 1 and Supplementary Figs. S2 to S4 in II) as well as numerical analysis of *Hin*dIII and *Eco*RI ribotypes (Supplementary Fig. S5 in II). The results obtained indicated that DSM 16698^T, AD5 and the related porcine lactobacilli strains constitute a single species, *L. amylovorus*, and that the name *L. sobrius* is a later synonym of *L. amylovorus*.

The classification of *L. sobrius* (Konstantinov et al., 2006) was based on DNA-DNA hybridization values, 16S rRNA gene sequence similarity, the ability to ferment

D-raffinose and FOS and to grow at 45°C. In our study, DNA-DNA hybridization values unambiguously indicated that all the studied strains belong to *L. amylovorus*. This species-level conclusion could be supported by numerical analysis of ribotypes, sequence comparison of two housekeeping genes (*pheS* and *rpoA*) and also by 16S rRNA gene sequence analysis. The 16S rRNA gene sequence published in the previous study (Konstantinov et al., 2006), distuinguishing the two species of *L. sobrius* and *L. amylovorus,* was revised later by Konstantinov et al. (2006). The revised sequence possesses much higher similarity with corresponding sequence of the *L. amylovorus* type strain (99.9%).

5.4. Characterization of the genes encoding the S-layer protein of *L. brevis* ATCC 14869 (III)

L. brevis strains ATCC 8287 and ATCC 14869, originating from plant and human faeces, respectively, show differences in their ability to bind epithelial cells (M. Jakava-Viljanen and A. Palva, unpublished results). Our adhesion studies indicate that the neotype strain of *L. brevis*, ATCC 14869, binds more strongly to human enterocytes *in vitro* than *L. brevis* ATCC 8287, and even the binding of ATCC 14869 to pig intestinal epithelial cells was found. To resolve the basis of these differences and to reveal whether the *L. brevis* S-layer proteins have structural similarity in their epithelial cell binding domains, the S-layer protein gene content and the *slp* gene expression in ATCC 14869 were characterized in detail in study III.

5.4.1. The S-layer protein profile

During the study, *L. brevis* ATCC 14869 cells were observed to form two colony types on MRS plates when growing under aerobic conditions for longer periods (Fig. 1 in III), whereas under anaerobic conditions only S-type colonies were formed.

This phenomenon has not earlier been described for this *L. brevis* neotype strain. Identical 16S ribosomal DNA (rDNA) sequences with *L. brevis* identity from the cells of both colony types excluded the possibility of strain contamination. In association with the morphological change of colonies, a switch of the *slp* gene type expressed was also observed. Two putative S-layer protein bands of 50 and 43 kDa were detected by whole-cell SDS-PAGE analyses from R-colony type cells reproduced on MRS plates and cells grown under aeration in MRS broth. The 50 kDa band was predominant in cells of the S-colony type and in cells grown anaerobically in MRS broth. Electron microscopy (EM) analysis revealed the presence of only a single S-layer with an oblique structure on top of the peptidoglycan layer on the cells of both colony types (Fig. 3 in III). Thus, the EM data do not explain the difference in the colony morphology but suggest that the S-layer structures are not directly involved.

5.4.2. Sequence analysis of the *slp* genes

PCR oligonucleotides were designed according to the N-terminal amino acid sequences from the purified 50 and 43 kDa S-layer proteins and their tryptic peptides and the PCR products obtained were sequenced and used as probes in Southern hybridizations with different restriction enzyme digestions. Based on the blotting data obtained, *Hin*dIII-cut chromosomal DNA was ligated with the *Hin*dIII Vectorette units, and PCR primers specific for the isolated gene and the Vectorette system were used to amplify and sequence the *slp* gene regions.

In the amino acid sequence predicted from the *orf* expected to encode the 50-kDa S-layer protein, the amino acid sequences of the intact N-terminus and tryptic peptides of this protein could be found, and the associated gene was designated *slpB*. The *slpB* gene was found to consist of 1449 nucleotides and to have the capacity to encode a polypeptide of 483 aa with a signal sequence of 30 aa. The molecular weight of the mature SlpB (48 kDa) was found to be in good agreement with the molecular mass of the 50 kDa protein estimated by SDS-PAGE. Sequence analysis of the *slpB* revealed

the presence of two putative promoter regions, a possible RBS and a putative rho-independent type transcription terminator sequence.

Downstream of the *slpB* sequence, another putative *slp* gene was found and designated *slpC*. The *slpC* gene, separated from *slpB* by 347 nucleotids, was found to consist of 1383 nucleotides with a capacity to encode a polypeptide of 461 aa. A predicted signal sequence of 30 aa was identified from the deduced *slpC* encoded polypeptide, SlpC, indicating that the *slpC* gene has a coding capacity for a mature protein of 46 kDa. Sequence analysis revealed the presence of two putative -10 regions for a promoter and a possible RBS, but the gene was not followed by transcription terminator-like sequences.

Because the predicted DNA sequence and coding capacity of the *slpC* did not correspond to the N-terminal sequence and size of the 43 kDa protein S-layer protein from R-colony type cells, primers were designed for the isolation of the third gene, *slpD*, from the Vectorette library.

The *orf* of the *slpD* gene was shown to consist of 1239 nucleotides and to encode a polypeptide of 413 aa with a signal sequence of 30 aa. The molecular weight of the mature SlpD (42 kDa) was found to be in good agreement with the molecular mass of the 43-kDa protein estimated by SDS-PAGE. Two putative -10 regions for a promoter and a possible RBS were identified from the upstream region of *slpD* and downstream of the stop codon a putative terminator sequence could be recognized. Southern blotting and PCR analysis revealed that the *slpD* gene is not closely linked to the *slpB*-*slpC* gene region.

Several lactobacilli possess multiple S-layer protein genes, which can be differentially or simultaneously expressed. The genetic organization of the three *L. brevis* ATCC 14869 *slp* genes is distinct from that described so far for other lactobacilli (Vidgrén et al., 1992; Boot et al., 1993; Callegari et al., 1998; Sillanpää et al., 2000; Altermann et al., 2005; Hagen et al., 2005). In *L. brevis*, two out of the three S-layer protein genes, *slpB* and *slpC*, are located adjacent to each other in parallel orientation whereas the third gene,

slpD, is not closely linked to the *slpB–slpC* gene region. The *slpC* gene was shown to be silent under the growth conditions tested. From *L. brevis* ATCC 8287, only one S-layer protein gene has been found, *slpA* (Vidgrén et al., 1992; Palva et al., unpublished results). In *L. acidophilus* ATCC 4356, the two S-layer protein genes, *slpA* and *slpB*, are in opposite orientation to each other and interspaced with a 3 kb DNA-region (Boot et al., 1996). The spacing and orientation of the *L. crispatus* S-layer protein genes, *cbsA* and *cbsB*, have not yet been deduced (Sillanpää et al., 2000). Based on the current data on the genetic organization of the *Lactobacillus* S-layer protein genes, they thus generally seem to be located in a rather close proximity to each other.

Despite the high similarity of the amino acid composition of all known S-layer proteins, the overall sequence similarity is, however, surprisingly small even between the *Lactobacillus* S-layer proteins. Homology comparisons of the *L. brevis* Slp proteins revealed that the intraspecies identity was mainly restricted to the N-terminal regions of these proteins, whereas the C-terminal regions were rather divergent. By pairwise protein sequence alignments computed to the *L. brevis* Slp proteins a few middle and C-terminal regions with considerable sequence similarity have, however, been found between the SlpB and SlpD proteins (Åvall-Jääskeläinen and Palva, 2005). The amino acid sequence identities of the *L. brevis* ATCC 14869 Slp proteins to the SlpA protein of *L. brevis* ATCC 8287 were lower than expected and negligible compared to other S-layer proteins of the *Lactobacillus* species studied. Due to the dissimilarity between the S-layer proteins of the two studied *L. brevis* strains and also due to the dissimilarity between the ATCC 14869 Slp and other lactobacillar S-layer proteins, the location of the putative receptor-binding domain and the cell wall binding domain of the ATCC 14869 Slp protein/s cannot be directly determined. On the other hand, sequence alignments between the S-layer proteins of *L. crispatus* and *L. acidophilus* and *L. helveticus* have shown that the C-terminal sequences between the S-layer proteins of these lactobacilli are highly similar (Åvall-Jääskeläinen and Palva, 2005) and contain a putative carbohydrate-binding consensus sequence, suggested to be involved in cell wall binding (Smit et al., 2001). For the S-layer proteins of *L. brevis* ATCC 14869 the adhesin function and location of the adhesion domain needs to be experimentally confirmed.

5.4.3. Expression studies on the *slp* genes

The expression of the *slp* genes of *L. brevis* ATCC 14869 was studied as a function of growth. Northern blot analyses were performed to investigate expression of all the three *L. brevis* ATCC 14869 *slp* genes under aerated and anaerobic growth conditions. Under both conditions, the *slpB*-specific probe detected an approximately 2.0-kb transcript, confirming the monocistronic nature of *slpB* transcripts. The *slpD*-specific probe detected an approximately 1.9-kb transcript only from total RNA isolated from cells grown under aerated conditions. The size of *slpD* transcripts indicated that the *slpD* gene is also monocistronic. With primer extension analysis, a transcription start site of the *slpD* gene could also be localized. Under aerated growth conditions, both *slpB* and *slpD* were found to be expressed, whereas under anaerobic growth conditions only expression of *slpB* was detected. Under the growth conditions tested, *slpC* was found to be a silent gene.

The phase variation mechanisms of S-layer proteins described so far are essentially based on DNA rearrangements (Åvall-Jääskeläinen and Palva, 2005). To study whether the activation of *slpD* gene expression takes place by chromosomal rearrangement, a real-time quantitative PCR analysis was performed with primers spanning the *slpD* promoter region and with chromosomal DNA from ATCC 14869 cells grown under aerated or anaerobic conditions. The result indicated that chromosomal rearrangements are not likely to be involved in the regulation of the *L. brevis slpD* gene expression.

In accordance with Northern data, expression studies with a PepI reporter protein under the control of the *slpD* promoter showed that under aerated conditions the specific PepI activities were growth stage dependent. Under anaerobic conditions no similar response was observed and the specific PepI activities remained low.

The results obtained in this study indicated that the variation in S-layer protein content in *L. brevis* ATCC 14869 occurs at the transcriptional level rather than with DNA rearrangements. The Northern analyses indicated that *slp* gene expression is regulated at the transcriptional level. This was also confirmed by expressing reporter protein PepI

under the control of the *slpD* promoter. Furthermore, we showed by quantitative real-time PCR analysis of the chromosomal DNA from cells grown under aerated or anaerobic conditions that there are no conformational changes in the known promoter region of *slpD*. When SlpD is produced, the amount of SlpB is lowered and the level of *slpB* transcripts is also somewhat decreased. It suggests that the variation in the S-layer protein content takes place by a unique mechanism involving activation of *slpD* transcription in the exponential growth phase by a soluble factor as a result of an environmental change. How this response is triggered, and what all the participating components are remains to be elucidated. It is also unclear whether the variation in the colony forms and *slp* gene expression are connected by the same regulon or whether they are independent events. Furthermore, it cannot yet be predicted whether the change in redox potential or amount of dissolved oxygen has a direct effect on the induction of *slpD* expression or whether some other changes in environmental factors under these conditions affect the control of *slpD* transcription. Most likely, a stress-related cascade of events may be involved.

5.5. Characterization of the adhesin of *E. coli* F18 fimbriae (IV)

F18 fimbriae of *E.coli* mediate bacterial adherence to the microvillus of porcine intestinal epithelial cells. A protein known as adhesin is responsible for this specific binding. A previous study suggests that the adhesin molecule of F18 fimbriae is either FedE or FedF (Imberechts et al., 1996). In this study, the unknown region of the *E. coli fed* gene cluster was sequenced and the adhesion of F18 fimbrial strain (ERF2055) to isolated porcine epithelial cells *in vitro* was confirmed. Furthermore, purified FedF and FedE as fusion proteins with maltose binding protein (MBP) were used for raising antisera for adhesion studies. In addition, using indirect immunofluorescence microscopy and adhesion inhibition tests, the FedF protein as the adhesin of F18 fimbriae was confirmed.

5.5.1. Sequencing of the *fed* gene cluster

F18 fimbriae were purified from *E. coli* F107/86 (Bertschinger et al., 1990). The genes involved in the biosynthesis of F18 fimbriae were revealed from plasmid pIH120, which was constructed by Imbrechts et al. (1992). The entire *fed* gene cluster was sequenced from plasmid pIH120 in study IV, completing the missing sequence data of the *fed* gene cluster. Two unknown open reading frames (ORF), designated *fedB* and *fedC* (accession no. AF222806), were found between *fedA* and *fedE*. On the basis of the homology alignment analyses performed, FedB is suggested to be the usher and FedC the chaperone of the F18 fimbria.

5.5.2. Adhesion studies

The adhesion of a reconstructed F18 fimbrial strain (ERF2055) to isolated porcine jejunal and ileal epithelial cells *in vitro* was confirmed, and the attachment of the F18 fimbrial *E. coli* strain was found to be mediated by FedF protein. Epithelial cells were isolated from the small intestine (jejunum and ileuml) of an 8-week old piglet and microscopic examination of the adherence of $F18^+$ *E. coli* (ERF2055) to the microvilli of the epithelial cells was performed. To obtain a semi-quantitative estimation of the level of adhesion, the number of bacteria adhering to 15 randomly chosen epithelial cells was counted. The average numbers of ERF2055 bacteria adhering per jejunal or ileal cell when the bacteria were preincubated with different antisera, which had been raised in rabbits or mice and diluted in phosphate-buffered saline, are listed in Table 1 in IV. Representative pictures are also shown for each adhesion analysis in Fig. 3 and Fig. 4 in IV. Abolishment of the adhesion potential of ERF2055 cells was observed after preincubation of ERF2055 cells with MBF-FedF-specific antibodies or antibodies directed against the entire F18 fimbria. In contrast, antibodies to MBP-FedC were not able to inhibit the adhesion of ERF2055 cells, even though a minor decrease in adhesion capability was found (Table 1 in IV). These results confirmed that from the antisera directed against FedF subunit, only MBP-FedF antibodies were able to

efficiently inhibit the adherence of the F18 fimbriae-expressing strain (ERF2055). As expected, a reduction in the adhesion capability of ERF2055 cells, reincubated with antibodies raised against whole F18 fimbriae could also be demonstrated.

A second adhesion study was set up with purified proteins to determine whether the MBP-FedF fusion protein could adhere to isolated porcine epithelial cells when produced without its chaperone (FedC). In addition, MBP-FedE and MBP were also tested for adherence as negative controls. The attchment of purified fusion proteins was detected with fluorescent microscopy after incubation with rabbit anti-MBP antiserum and FITC-labelled anti-rabbit antibodies. The epithelial cells incubated in the presence of MBP-FedF exhibited bright fluorescence (Fig. 5A in IV), whereas epithelial cells incubated with equal amounts of MBP-FedE (Fig. 5C in IV) or MBP (data not shown) showed no fuorescence. This strongly suggested that the FedF protein represents the adhesin of the *fed* gene cluster encoding the F18 fimbria and also indicated that the FedF produced as a fusion protein with MBP without its chaperone retained its capacity to bind to epithelial cells.

6. CONCLUSIONS AND FURTHER ASPECTS

Slps of lactobacilli have been shown to confer tissue adherence. In order to find the potential porcine-specific S-layer carrying strains for later use as vaccine vectors and/or probiotics in pigs, in total 99 strains, putatively belonging to the genus *Lactobacillus*, were isolated as pure cultures and the adhesiveness of the putative S-layer-expressing lactobacilli to intestinal tissues was investigated. From the all isolates tested, only 8% of the strains produced S-layer proteins. It is likely that the amount of S-layer carrying porcine lactobacilli is relatively low. Their taxonomic identification, based on polyphasic taxonomy study, revealed that the eight S-layer protein-producing strains belonged to the species *L. amylovorus* and *L. crispatus*. In addition, *L. sobrius* strains DSM 16698^T and AD5 were found to belong to the species *L. amylovorus.* Thus, *L. sobrius* is a later synonym of *L. amylovorus*. No *L. acidophilus* and *L. brevis*, known to exhibit an S-layer structure and shown to be one of the dominant *Lactobacillus* species in pigs in earlier studies, were found in this study. Our result suggests that either *L. amylovorus* is the dominant S-layer carrying *Lactobacillus* in pigs or alternatively this finding may reflect the effect of feeding constituents on the enrichment of a certain *Lactobacillus* species.

We found that the SDS-extractable protein profile, the size of the S-layer protein and binding capability of the strains varied greatly, even among the isolates belonging to the same *Lactobacillus* species. PCR isolation of S-layer protein genes suggests the presence of more than one *slp* gene, at least in some of the strains. Removal of the intact Slps from the bacterial surface by extraction with GHCl reduced the adhesion of some strains to fibronectin and laminin. This is in accordance with the observation that S-layer subunits indeed are adhesion proteins and one of the key adhesion factors of the cell.

Our adhesion studies indicated that the neotype strain of *L. brevis*, ATCC 14869, adheres strongly to human and pig intestinal epithelial cells *in vitro*. To study whether the S-layer protein of ATCC 14869, like that of *L. brevis* ATCC 8287 characterized earlier, mediates adhesion and whether the *L. brevis* S-layer proteins have structural similarity in their epithelial binding domains, we characterized two new S-proteins and

three new *slp* genes. The proteins have distinct differerences when compared to the S-layer proteins from *L. brevis* ATCC 8287 and other lactobacilli previously described. Under different growth conditions, *L. brevis* strain 14869 was found to form two colony types, smooth (S) and rough (R), and to express the S-layer proteins differently, a phenomenon not earlier described. Most likely to adapt to different stress factors caused by drastic changes in the environmental conditions, the bacteria may express alternative S-protein genes.

In the infection process, F18 fimbriae of *E. coli* mediate bacterial adherence to the the microvillus of porcine intestinal epithelial cells. In this study, the adhesin of *E. coli* F18 fimbriae was established to be FedF. Moreover, specific adhesion to enterocytes was shown with purified FedF-maltose binding protein. Therefore, oral immunization of piglets with recombinant FedF could be considered. The S-layer could provide a superior expression level and surface density of the required FedF antigen. In cases where an antigen providing the binding function to the epithelium, like the *E. coli* F18 receptor-binding domain, the role of binding provided by the S-layer carrier molecule may be of less importance. With an antigen having no binding capacity, the role of the S-layer binding property to the epithelium may also become important, for example, in the prevention of colonization of the invading pathogen by competitive exclusion, in addition to eliciting antibody responses against the pathogen.

This study is part of a larger project aimed at developing live oral LAB vaccines. Vaccination with live recombinant bacteria, producing certain antigens, is an attractive alternative to the traditional vaccination methods. The advantages in employing LAB as vaccine vectors include the claimed health-promoting properties (Vaughan et al., 1999) and the intrinsic adjuvant activities (Seegers, 2002) possessed by several strains of LAB, the GRAS status of LAB (Adams and Marteau, 1995) as well as the lack of lipopolysaccharides in the cell wall of LAB, eliminating the risk of endotoxic shock (Mercenier et al., 2000). LAB strains carrying adhesion molecules from pathogens on the surface may, in addition to eliciting local and humoral immune responses against the pathogen, also hinder colonization of the invading bacteria by competitive exclusion.

F18 fimbrial *E. coli* strains cause PWD and ED in pigs that often are fatal to weaned pigs and are responsible for considerable economic losses in the pig breeding industry (Verdonck et al., 2002). So far, commercial vaccines against infections caused by the F18 fimbriae carrying *E. coli* strains are lacking. The practice of preventing ED and PWD by administering antimicrobials or feed additives no longer meets the criteria for rational use of antibiotics in production animals due to the development of drug resistance and harmful residues in meat. A need therefore exists to develop alternative treatments for such diseases. Vaccination with live recombinant bacteria, producing specific antigens, is an attractive alternative to the traditional vaccination methods and could be utilized in the prevention of infections caused by $F18^+$ *E. coli*.

The receptor binding domain of the FedF adhesin of *E. coli* strains carrying F18 fimbriae has been chosen as the first target antigen to be expressed in *L. brevis* in our laboratory. By surface displaying epitopes or antigens as part of an S-layer, up to 5×10^5 epitope monomers can be obtained to surround a single *Lactobacillus* cell, making such lactobacilli very attractive candidates for mucosal vaccine delivery vectors. Immunization studies with *Lactobacillus* S-layer antigen constructs are still lacking, but the results of the few immunization studies utilizing recombinant S-layer proteins of other bacteria (Umelo-Njakka et al., 2001, Riedmann et al., 2003) further encourage the development of *Lactobacillus* S-layer based antigen carriers.

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